

Phosphorylation of the Sic1 Inhibitor of B-Type Cyclins in *Saccharomyces cerevisiae* Is Not Essential but Contributes to Cell Cycle Robustness

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Manuscript received March 20, 2007

Accepted for publication April 24, 2007

ABSTRACT

In budding yeast, B-type cyclin (Clb)-dependent kinase activity is essential for S phase and mitosis. In newborn G₁ cells, Clb kinase accumulation is blocked, in part because of the Sic1 stoichiometric inhibitor. Previous results strongly suggested that G₁ cyclin-dependent Sic1 phosphorylation, and its consequent degradation, is essential for S phase. However, cells containing a precise endogenous gene replacement of *SIC1* with *SIC1-OP* (all nine phosphorylation sites mutated) were fully viable. Unphosphorylatable Sic1 was abundant and nuclear throughout the cell cycle and effectively inhibited Clb kinase *in vitro*. *SIC1-OP* cells had a lengthened G₁ and increased G₁ cyclin transcriptional activation and variable delays in the budded part of the cell cycle. *SIC1-OP* was lethal when combined with deletion of *CLB2*, *CLB3*, or *CLB5*, the major B-type cyclins. Sic1 phosphorylation provides a sharp link between G₁ cyclin activation and Clb kinase activation, but failure of Sic1 phosphorylation and proteolysis imposes a variable cell cycle delay and extreme sensitivity to B-type cyclin dosage, rather than a lethal cell cycle block.

B-TYPE cyclin regulation is central to cell cycle control. In budding yeast, DNA replication and entry into mitosis are driven by B-type cyclins (Clb's) activating the cyclin-dependent kinase Cdc28, and mitotic Clb-Cdc28 is antagonistic to mitotic exit (NASMYTH 1996). The Cdk activity cycle is interdigitated with regulation of the anaphase-promoting complex (APC) (ZACHARIAE and NASMYTH 1999), and multiple oscillatory mechanisms collaborate to provide alternate periods of low and high Clb-Cdk activity (MORGAN and ROBERTS 2002; CROSS 2003). The lowest period of Clb-Cdk activity is in newborn G₁ cells. In G₁, Cdh1 promotes APC-dependent ubiquitination and proteolysis of mitotic Clb's such as Clb2 (SCHWAB *et al.* 1997; VISINTIN *et al.* 1997). Independently, activation upon mitotic exit of *SIC1* transcription leads to accumulation of Sic1 protein, a potent stoichiometric inhibitor of Clb-Cdk (SCHWOB *et al.* 1994; KNAPP *et al.* 1996). An added mechanism is low transcription of all *CLB* genes in early G₁ (WITTENBERG and REED 2005). Thus Clb kinase activation in early G₁ is stringently regulated.

In late G₁, a burst of gene expression dependent on the factors SBF/MBF is triggered by Cln3-Cdk (WITTENBERG and REED 2005). Among the targets of SBF/MBF are the G₁ cyclins Cln1 and Cln2. These cyclins form a Cdk complex that is insensitive to Sic1 and Cdh1 and that initiate Sic1 and Cdh1 phosphorylation on multiple sites (there are 9 Cdk sites in Sic1 and 11 in Cdh1).

Sufficient phosphorylation of these proteins results in their inactivation. Phosphorylated Cdh1 loses the ability to interact with the APC (ZACHARIAE *et al.* 1998). Phosphorylated Sic1 is specifically recognized by the ubiquitination E3 complex SCF-Cdc4, leading to ubiquitination and proteolysis of phosphorylated Sic1 (VERMA *et al.* 1997a; NASH *et al.* 2001). While there is no unique combination of sites in Sic1 required for Cdc4 binding, at least 6 of the 9 sites may need to be phosphorylated for efficient binding (VERMA *et al.* 1997a; NASH *et al.* 2001; ORLICKY *et al.* 2003).

Upon Clb-Cdk activation, Sic1 and Cdh1 phosphorylation can be carried out by Clb-Cdk instead of Cln-Cdk; thus Cln-Cdk can be viewed as flipping a switch that allows a transition between two otherwise stable states, one of low and one of high Clb-Cdk activity (MORGAN and ROBERTS 2002; CROSS 2003).

Sic1 binds tightly to the Clb-Cdc28 complexes required for DNA replication (MENDENHALL 1993; SCHWOB *et al.* 1994), so the essentiality of removal of Sic1 is probably dependent on the relative stoichiometry of Sic1 and B-type cyclins if Sic1 were not degraded. Strains with temperature-sensitive mutations in SCF-Cdc4 components arrest in G₁ at high temperature with high levels of stable Sic1 and low Clb kinase activity. Deletion of the *SIC1* gene in these mutants allowed DNA replication, leading to the conclusion that the stable Sic1 was directly responsible for blocking DNA replication in the absence of SCF-Cdc4 (SCHWOB *et al.* 1994; KNAPP *et al.* 1996). In another approach, moderate (~1.5-fold) overexpression of a Sic1 mutant stabilized by four phosphorylation-site mutations blocked DNA replication in a G₁ block-release

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protocol; similar overexpression of wild-type Sic1 had no effect because the protein was ubiquitinated and degraded (VERMA *et al.* 1997a). These experiments led to the conclusion that at endogenous levels of Sic1 and B-type cyclins, Sic1 degradation is essential for DNA replication.

Sic1 stabilized by SCF inactivation is not in great excess for blocking S phase in *cdc4* mutants, since heterozygous *sic1/SIC1 cdc4/cdc4* diploids carried out DNA replication, unlike homozygous *SIC1/SIC1 cdc4/cdc4* diploids (KNAPP *et al.* 1996). Measurement of Sic1 and all B-type cyclins on a common scale indicated that, in a normal cell cycle, Sic1 did not accumulate in excess to peak B-type cyclin levels (CROSS *et al.* 2002). However, *CLB5* overexpression was reported not to accelerate DNA replication in G_1 cells due to the presence of Sic1, suggesting at least some excess of Sic1 over Clb5 in these conditions (SCHWOB *et al.* 1994).

cln1 cln2 cln3 cells, lacking all G_1 cyclins and normally blocked permanently in G_1 , are partially rescued by deletion of *SIC1* (TYERS 1996), suggesting that, in the *cln1 cln2 cln3* background, Sic1 is present at a high-enough level to titrate out any available Clb5 or other B-type cyclin; however, the absence of Cln3 and consequent absence of efficient *CLB5* transcription (WITTENBERG and REED 2005) makes this situation difficult to compare accurately to wild type.

The G_1 cyclins Cln1 and Cln2 probably carry out the bulk of physiological Sic1 phosphorylation; strains lacking *CLN1* and *CLN2* are highly sensitive to *SIC1* gene dosage (TYERS 1996). Strains lacking *CLN1* and *CLN2* probably degrade Sic1 slowly, and DNA replication is delayed until these cells reach a larger cell size; this delay is Sic1 dependent (DIRICK *et al.* 1995). This suggests that undegraded Sic1 delays DNA replication, but does not prove that undegraded Sic1 cannot block DNA replication indefinitely, since these cells do ultimately replicate DNA; however, the cells also likely ultimately degrade Sic1, probably due to Sic1 phosphorylation mediated by other cyclins (NISHIZAWA *et al.* 1998; MOFFAT and ANDREWS 2004).

Sic1 mutants with phosphorylation sites removed are as effective at Clb5-Cdc28 kinase inhibition as wild type (NASH *et al.* 2001). In contrast, phosphorylation is absolutely required for binding Cdc4 (VERMA *et al.* 1997a; NASH *et al.* 2001). The N-terminal region of Sic1 containing multiple phosphorylation sites is necessary and sufficient for ubiquitination, whereas the C-terminal region is necessary and sufficient for Clb5 binding (VERMA *et al.* 1997b). Indeed, only the C-terminal 70 amino acids of Sic1 are required for *in vivo* cell cycle inhibition upon overexpression (HODGE and MENDENHALL 1999). Thus, the sole function of the phosphorylation sites may be to promote cell-cycle-regulated Sic1 degradation.

In an interesting parallel to Sic1, the mammalian Cdk inhibitor p27, which accumulates to high levels in G_1 , is also degraded following SCF-dependent ubiquitination, which is dependent on Cdk phosphorylation of three-

nine 187. While this degradation could contribute to the G_1/S transition, the T187A mutation in p27 does not cause a significant cell cycle block; however, this mutant revealed a previously unappreciated G_1 mode of p27 degradation (MALEK *et al.* 2001). Thus, the consequence of complete stabilization of p27 remains unknown.

Here, we characterize a precise gene replacement of *SIC1* with a mutant allele lacking all nine Cdk sites, *SIC1-OP*, to rigorously test the proposed essentiality of Sic1 phosphorylation and the ensuing proteolysis (SCHWOB *et al.* 1994; VERMA *et al.* 1997a) at endogenously controlled levels and in an otherwise wild-type background.

MATERIALS AND METHODS

Plasmids: Standard methods were used throughout. Starting materials for plasmid constructions were the following: MT2728, *GAL-SIC1-OP* (NASH *et al.* 2001); MT907, *EcoRI-BglII* fragment containing the wild-type *SIC1* gene in RS316 (from M. Tyers); MDM168, *GAL-SIC1* in Y1plac204 (from A. Amon); RD609, *GAL-SIC1-del3P* (VERMA *et al.* 1997a) from R. Verma; and plasmids containing *GAL-SIC1-GFP* with and without Lys-Arg mutations in N-terminal Sic1 ubiquitination sites (PETROSKI and DESHAIES 2003a,b) (from R. Deshaies). The *EcoRI-HpaI* fragment from MT907 was subcloned into MDM168, replacing the *GAL1* promoter with the wild-type *SIC1* promoter. *SIC1-OP* was introduced in place of *SIC1-wt* by amplifying the endogenous promoter sequence from MT907 and the *SIC1-OP* sequence from MT2728 by polymerase chain reaction (PCR), combining the two PCR products by splice-overlap PCR and subcloning an *EcoRI-HpaI* fragment into MDM168. All cloned PCR products were sequenced. *SIC1-wt* and *SIC1-OP* inserts were subcloned into RS406, yielding FC667 and FC663. The *SpeI-HpaI* fragment from RD609 containing T33A, S76A, was subcloned into FC667 to reconstruct the four-site phosphorylation-site mutant (*SIC1-del3P*) under control of the endogenous *SIC1* promoter in FC672. (One difference was that *GAL-SIC1-del3P* in RD609 was T2A T5GP, while FC672 was T2A T5A.) FC675 (*SIC1-2P*) was made by recombining FC663 and FC667 at a *SpeI* site, resulting in restoring T2 and T5 phosphorylation sites to *SIC1-OP* in FC663. Fusions of *SIC1* and mutant derivatives to GFP were carried out by subcloning a *SIC1-GFP* fragment derived from *GAL-SIC1-GFP* plasmids (PETROSKI and DESHAIES 2003a) into *SIC1* plasmids. *SIC1* lacking six N-terminal ubiquitin-acceptor lysines (*SIC1-KON*: R32K, R36K, R50K, R53K, R84K, R88K) was constructed by subcloning a *SpeI-HpaI* fragment from the *GAL-SIC1-KON* plasmid (PETROSKI and DESHAIES 2003a) into FC667.

Strain constructions: A *swi5::kanMX sic1::HIS3* strain (W303 background) was constructed and transformed with *Pf1FI-cut* FC663 (targeting integration to the *SIC1* promoter 5' to *sic1::HIS3*, creating *SIC1-OP::URA3::sic1::HIS3*). Transformants were colony purified and Ura⁻ popouts selected on 5-FOA medium. His⁻ popouts were candidates for being *SIC1-OP* exact integrants. These popouts were confirmed by Southern blotting and by PCR amplification followed by sequencing across the entire locus, including the entire 5' and 3' noncoding regions. No mutations were found other than the expected phosphorylation-site mutations (and a deletion relative to the standard genomic sequence of one E residue from a poly(E) stretch in Sic1; this deletion is found in all our clones of *SIC1*). Similar methods were used for genomic introduction of *SIC1-5P*, *SIC1-2P*, and *SIC1-KON*; correct integration was confirmed by restriction fragment length polymorphism or sequence analysis of diagnostic PCR

products. Protein A-tagged versions (*SIC1-OP-PrA*, *SIC1-5P-PrA*, *SIC1-KON-PrA*) were constructed by similar methods in a *swi5::kanMX SIC1-ut-PrA::HIS3MX* background, containing the *SIC1* gene endogenously tagged with protein A; this is a functional fusion, described previously (Cross *et al.* 2002). In this case, FOA-R His⁺ popouts were tested by restriction digestion and sequencing of diagnostic PCR products.

Other strain constructions employed standard yeast mating and tetrad analysis. *CLN2pr-GFP* (the endogenous *CLN2* promoter driving destabilized GFP, with a functional copy of *CLN2* present in tandem) was described previously (MATEUS and AVERY 2000; BEAN *et al.* 2006). *GALS-CLB5* was from J. BLOOM (unpublished results) and *GALS-CLB2* was from C. LOOKINGBILL (unpublished results); the *GALS* promoter is a weakened galactose-regulatable promoter that provides tight regulation without strong overexpression (MUMBERG *et al.* 1995). Viability analysis of various genotypes in tetrad analysis was carried out by assuming a 2:2 segregation of markers to assign genotypes to inviable segregants. Only tetrads for which genotypes could be assigned to all spores, viable and inviable, were used for the quantitation. In this analysis, very tiny colonies (too small to genotype by replica plating) were scored as “inviable.” In some cases, *SIC1* genotypes were confirmed by PCR and restriction digestion to check for phosphorylation-site mutations.

Time-lapse fluorescence microscopy: We used a Leica DMIRE2 inverted motorized fluorescence microscope, with HCX Plan Apo, $\times 100$, numerical aperture 1.40, oil immersion objective, in a heated 30° incubation chamber, imaging cells on agar slabs with a Hamamatsu ORCA ER 1394 digital CCD camera with the gain set to 50 (scale 0–250) for GFP images. We used Image Pro Plus 4.5 IPP to adjust brightness and contrast of phase and fluorescence images, to resize the phase image to the same size as the binned fluorescent images, to overlay phase and fluorescence images to create false-colored composites (phase, white; GFP, green), and to readjust brightness and contrast of the phase and fluorescence channels of the composite image (no nonlinear adjustments made during processing). For Sic1-GFP images, an additional adjustment in brightness was uniformly applied to all images in Photoshop, to better document the Sic1-wt-GFP signal. *CLN2pr-GFP* contained yeast-enhanced GFP3; *SIC1-GFP* contained GFP(S65T,Q80R). Data collection and analysis using *CLN2pr-GFP* were done as described (BEAN *et al.* 2006), except that signal intensity and signal:noise ratio were enhanced by 2×2 binning of CCD camera pixels before data collection (our unpublished data).

For analysis of *CLN2pr-GFP* movies, assignment of budding times and genealogy (assignment of bud to a mother cell) was done manually using a custom-designed graphical user interface. All subsequent analysis of *CLN2pr-GFP* expression (standardized peak intensity and duration, time from budding to peak expression), cell cycle times (intervals between successive mother-bud emergences), and cell sizes at budding (pixel areas) were carried out completely automatically using the image and data analysis software described previously (BEAN *et al.* 2006), with the addition of a new software routine to determine the histograms of GFP peak widths (time between half-maxima rising and falling) for all defined peaks. Data points >3 SD from the mean were removed before calculating statistics in Table 1, to avoid outlier bias (this correction typically removed zero to two events and had no effect on the conclusions drawn). *CLN2pr-GFP* peak amplitudes refer to average pixel intensities over the segmented cell border, and all values are standardized to a reference wild type run in parallel in every experiment. The peaks are the maxima of smoothing spline fits, after trough-to-trough background subtraction, as described (BEAN *et al.* 2006). *CLN2pr-GFP* peak width is defined as the time from the rising to the falling attainment of 50% of the peak level

in the background-subtracted spline fit. Cell size at budding is defined as the number of pixels in the segmented mother-cell boundary at the time that bud emergence was scored. Mother-cell cycle time is defined as the interval between successive budding of a mother cell. The former statistics were all determined automatically by the automated data analysis software from the complete data set (BEAN *et al.* 2006). Bud long axis/short axis ratio was determined using images from the same movies and length analysis was determined using ImageProPlus, measuring bud dimensions 60 min after bud emergence.

Nuclear residence of Sic1-GFP and bud emergence were scored manually from composite-phase/fluorescence movies, as described previously for Whi5-GFP (BEAN *et al.* 2006). Sic1-wt-GFP signal was low and somewhat variable; occasional cells in which a Sic1-GFP signal could not be reliably scored (usually mother cells) were omitted from the analysis. The low signal also made scoring of the timing of Sic1-GFP nuclear residence ambiguous in some cases, potentially adding a few frames' error to this estimate. These scoring problems do not affect the qualitative conclusions reported. Sic1-OP-GFP and Sic1-5P-GFP gave a very bright signal that led to no scoring ambiguity.

Other methods: Flow-cytometry analysis (EPSTEIN and CROSS 1992) and Western blotting (CROSS *et al.* 2002) were done as described. Purification of GST-Sic1 was carried out by lysozyme-sonication lysis of *Escherichia coli* expressing GST fusion proteins (GST-Sic1 or GST-Sic1-OP; plasmids from M. Tyers) followed by purification on glutathione-sepharose and elution with glutathione for soluble preparations. Estimated concentrations of full-length soluble GST-Sic1 and GST-Sic1-OP (~ 0.6 mg/ml) were determined on the basis of amido-black-stained gel-transfer membranes compared to threefold serial dilutions of BSA standard (our unpublished data). For pull-down purification of Clb5-PrA with IgG-sepharose or with glutathione-sepharose carrying GST-Sic1 fusion proteins, yeast extracts were prepared from strain VAY79 (*cdc20 GALL-CDC20 CLB5-PrA::HIS3MX*) after block of the strain in glucose for 3.5 hr by breakage of cell pellets derived from 100 ml of culture in 400 μ l LSHNN buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 10% glycerol, 0.1% NP-40) with 400 μ l of glass beads by shaking in a FastPrep bead beater for two periods of 20 sec at a setting of 5, separated by 1 min on ice. Extracts were clarified by a 1-min microfuge spin and precipitated with bead-bound affinity reagent (GST-Sic1 or IgG) (1 hr on ice, followed by three washes in LSHNN, one wash in the same buffer with 250 mM NaCl, and, for kinase assays, one wash and resuspension in kinase buffer (10 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM DTT). For kinase assays, 15 μ l of IgG-bound Clb5-PrA (purified from ~ 20 ml of culture) were incubated on ice for 15 min with 600, 60, or 6 ng of GST-Sic1. Reaction mix (5 μ l) containing 2 μ g of histone H1, 5 μ M ATP, and tracer [³²P]ATP was added. Reactions were incubated for 10 min at 30°. The final concentration of Sic1 in the assays was estimated to be ~ 500 , 50, and 5 nM.

RESULTS

Expression of unphosphorylatable Sic1 from the endogenous promoter is not lethal, but results in a lengthened G₁: The expected lethality of endogenous expression of unphosphorylatable Sic1 (see Introduction) should be alleviated in the absence of the Swi5 transcription factor that activates *SIC1* expression, since Sic1-dependent failure to replicate in a *cdc4* background was rescued by *swi5* deletion (KNAPP *et al.* 1996). Therefore, we carried out a two-step gene replacement of *sic1::HIS3* with *SIC1-OP* [containing none of the nine

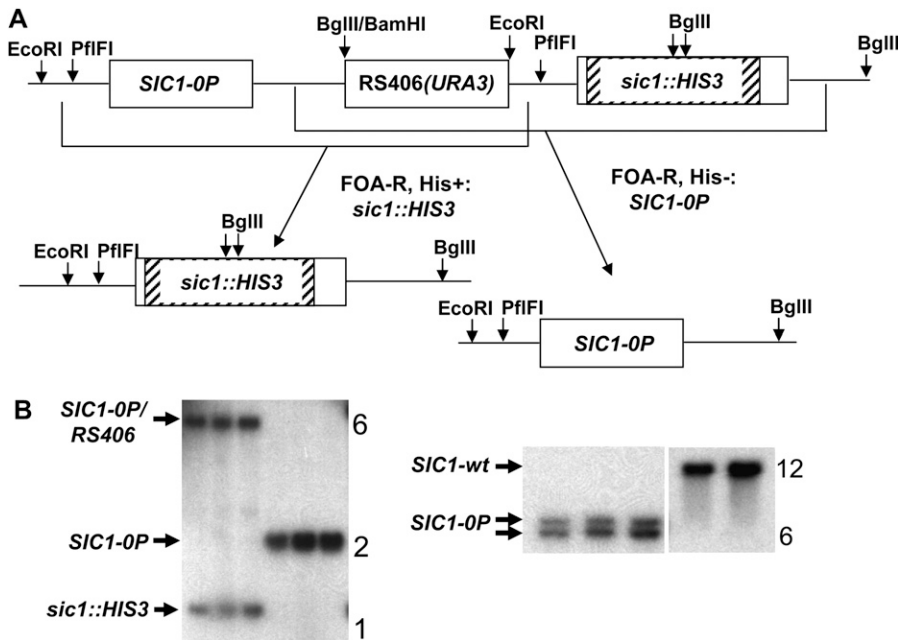


FIGURE 1.—Scheme for replacement of *SIC1* with *SIC1-OP*. (A) *SIC1-OP*, including a 5' promoter sequence, in the cloning vector RS406 (*URA3*) (plasmid FC663) was digested with *PflFI*, cutting in the *SIC1* promoter, and used to transform a *sic1::HIS3* strain. The expected resulting structure is shown on top (not to scale). Homologous recombination events resulting in *URA3*–popout derivatives were selected on the basis of 5-FOA resistance and were found to be a mix of His⁺ and His[–]. These were interpreted as being due to recombination in the regions indicated by brackets. (B) Selected Southern blot analysis: (Left) *EcoRI*+*BglII* digestion of DNA from three integrants of RS406-*SIC1-OP* at the *sic1::HIS3* locus (first three lanes) and FOA-resistant His[–] popouts derived from these integrants (second three lanes), probed with *SIC1* DNA from *EcoRI* to *KpnI* (at border of *HIS3* insertion in *sic1::HIS3*), confirming replacement of *sic1::HIS3*

with *SIC1-OP* in the His[–] popouts. (Right) DNA from the same three FOA-resistant His[–] popouts and two *SIC1-wt* controls digested with *ApaI* and probed with a *SIC1* coding sequence probe, confirming an *ApaI* site introduced at the S80A phosphorylation-site mutation (near the center of an ~12-kb genomic *ApaI* fragment containing *SIC1*). Deduced identities of the bands are indicated; band sizes are approximately as expected from known genomic sequence.

consensus Cdk sites in Sic1 (Nash *et al.* 2001) (Figure 1)] in a *swi5::kanMX* background. Gene replacements were confirmed by Southern blotting and by sequencing of PCR products spanning the entire locus (Figure 1; our unpublished data). This strategy results in exact replacement of the coding sequence of *SIC1* with *SIC1-OP*, under control of the endogenous promoter, with no associated vector or marker sequences. We crossed *SIC1-OP swi5::kanMX* to *sic1::HIS3 SWI5*, so that we could assess viability of *SIC1-OP SWI5* segregants (G418-S His[–]) among the progeny by tetrad analysis. In contrast to expectation, such progeny were not inviable; they were recovered at expected Mendelian proportions, exhibiting a moderately reduced growth rate, and an increased proportion of cells in G₁ as determined by flow-cytometry analysis (Figure 2).

SIC1 is essential in the absence of *CDH1*, an activator of the anaphase-promoting complex (SCHWAB *et al.* 1997; VISINTIN *et al.* 1997). To establish that *SIC1-OP* was functional, we crossed *SIC1-OP* strains to *cdh1::LEU2* strains. Viable *SIC1-OP cdh1::LEU2* segregants were readily obtained, while *sic1::HIS3 cdh1::LEU2* segregants constructed in parallel were inviable, as expected. Asynchronous *cdh1::LEU2* strains had a reduced G₁ population compared to wild type; *SIC1-OP* significantly increased G₁ in the *cdh1::LEU2* background (Figure 2), indicating that Sic1-OP restrains S-phase entry even in the absence of Cdh1.

Viability of cells expressing Sic1 lacking only some phosphorylation sites: Removal of as few as four of the nine Cdk sites from Sic1 can eliminate its interaction with SCF-Cdc4 (VERMA *et al.* 1997a; NASH *et al.* 2001).

Therefore, we carried out gene replacements of endogenous *SIC1* with the four-site mutant of VERMA *et al.* (1997a), called by them *SIC1-del3P*, which we have reconstructed under control of the endogenous promoter; we name this mutant here *SIC1-5P* for consistency, indicating the retention of five sites. This mutant was shown to possess full Cdk inhibitory activity and to completely escape ubiquitination by SCF-Cdc4 (VERMA *et al.* 1997a). We recovered viable *SWI5* strains with the *SIC1-5P* mutation as well as with numerous other combinations of phosphorylation-site mutations, including a mutant containing only the T2 and T5 sites (*SIC1-2P*) (our unpublished data).

Sic1-OP binds Clb5 and inhibits Clb5-associated kinase activity: We carried out bacterial expression and purification of recombinant GST fusion proteins containing Sic1-wt or Sic1-OP. We assessed the ability of the GST fusion proteins to stably bind to Clb5 (tagged with protein A); GST-Sic1-OP was equivalent to GST-Sic1-wt in these assays (Figure 3A), confirming previous findings that Clb5 binding is independent of phosphorylation, and indeed independent of the N-terminal regions containing the phosphorylation sites (VERMA *et al.* 1997a). We also observed essentially comparable inhibition of Clb5-associated kinase activity by soluble GST-Sic1-OP and GST-Sic1-wt (Figure 3B). Clb5 kinase inhibition assays of a number of phosphorylation-site mutants, including Sic1-OP, carried out using physiological concentrations of recombinant inhibitor and kinase, showed comparable inhibition by all Sic1 mutants to wild type (NASH *et al.* 2001); our results confirm this finding.

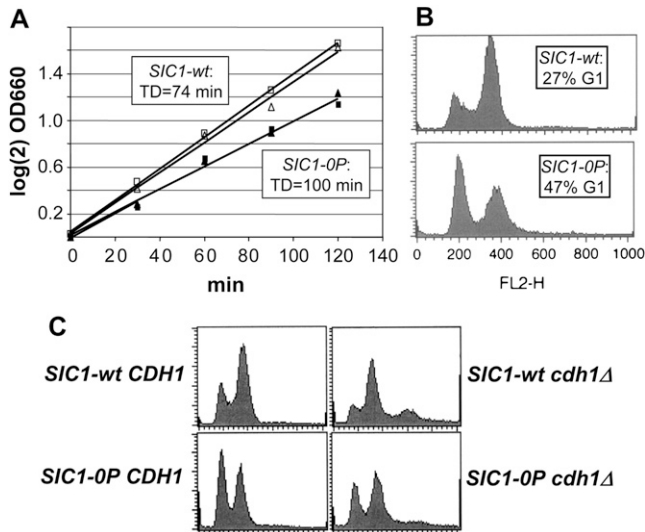


FIGURE 2.—*SIC1-OP* cells are viable with an elongated G_1 . (A) *SIC1-OP SWI5* and *SIC1-wt SWI5* strains were constructed and grown in YEPD at 30°. At intervals, the OD₆₆₀ was determined to measure the doubling times, indicated in the insets. (B) In the middle of the growth curve shown in A, samples were taken for flow-cytometry analysis. An approximate quantitation of the proportion of 1C DNA content (G_1) cells was made (insets). (C) A *SIC1-OP* strain was crossed to a *cdh1::LEU2 sic1::HIS3 pURA3-SIC1* strain, and tetrads were dissected. Flow-cytometry data from representative segregants (all lacking the *pURA3-SIC1* plasmid) are presented.

Sic1 phosphorylation-site mutants are stable throughout the cell cycle: Lack of cell cycle arrest with Sic1-OP or Sic1-5P at endogenous expression levels could be due to proteolysis of these proteins by a phosphorylation-independent mechanism. While no such mechanism was detected previously (VERMA *et al.* 1997a; NASH *et al.* 2001), these previous experiments depended upon Sic1 overexpression, which could have saturated this hypothetical phosphorylation-independent mechanism.

To test stability of unphosphorylatable Sic1 at endogenous expression levels, we constructed *SIC1-OP-PrA* and *SIC1-5P-PrA* at the endogenous locus, by integration excision at a previously characterized functional *SIC1-PrA* locus (CROSS *et al.* 2002). In these strains, we included a *GALS-CLB5* cassette. Wild-type Sic1-PrA exhibited a pattern of accumulation consistent with cell cycle regulation of its abundance (Figure 4): almost no Sic1-PrA accumulation in cultures delayed in S/ G_2 by Clb5 overexpression (on galactose medium, where *GALS-CLB5* was expressed) (JACOBSON *et al.* 2000), a moderate amount in normally cycling cultures (on glucose medium, without *GALS-CLB5* overexpression), and a high amount in cells blocked in G_1 by the mating pheromone α -factor. Sic1-5P-PrA and Sic1-OP-PrA, in contrast, were present at comparably high levels with all these treatments.

Upon release of the α -factor block, Sic1-wt-PrA was lost rapidly, around the time of bud emergence, reappearing faintly around the time of the succeeding mitosis. In contrast, Sic1-OP-PrA was stable throughout the cell cycle

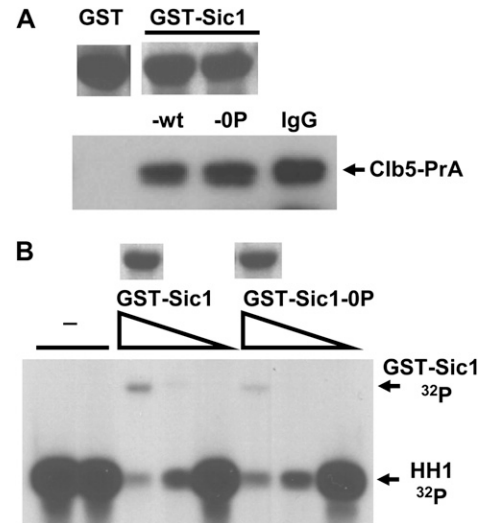


FIGURE 3.—Sic1-OP binds and inhibits Clb5-Cdc28. (A) GST, GST-Sic1, and GST-Sic1-OP were purified from *E. coli* on glutathione-sepharose beads. VAY79 (*cdc20 GAL-CDC20 CLB5-PrA*) was blocked in glucose medium to deplete Cdc20 and allow accumulation of high levels of Clb5-PrA. Extracts made from this culture were incubated for 1 hr on ice with glutathione-sepharose beads containing equivalent amounts of GST, GST-Sic1, or GST-Sic1-OP (parallel amido black stains for GST fusions at top of figure) or with IgG-sepharose beads to directly purify Clb5 via the PrA tag, and beads were washed by repeated centrifugation. Western blot analysis of bead-bound Clb5-PrA is shown. (B) Clb5-PrA was purified from cell extracts as in A using IgG-sepharose. Aliquots (15 μ l) of Clb5-PrA-IgG-agarose bead suspension in kinase buffer were incubated for 15 min on ice with no addition, with 1 μ l of glutathione elution buffer, or with glutathione elution buffer containing 10-fold serial dilutions of soluble GST-Sic1 fusions (an estimated addition of ~600, 60, and 6 ng of GST-Sic1 or GST-Sic1-OP; parallel amido black stains of GST fusions at top). Then 5 μ M ATP, 50 μ Ci 32 P-labeled ATP, and 2 μ g histone H1 were added (final 21 μ l). Reactions were incubated for 10 min at 30°, stopped with SDS sample buffer, and separated by gel electrophoresis before exposure to film to assess histone H1 kinase activity. Phosphorylation of GST-Sic1, and less efficient phosphorylation of GST-Sic1-OP, were also detected, as indicated.

(Figure 5). Cell cycle progression in the two cultures was similar on the basis of budding, nuclear division, Clb5-myc accumulation, and DNA replication (Figure 5). Sic1-5P-PrA gave essentially the same results as Sic1-OP-PrA with respect to protein stability and cell cycle progression in this protocol (supplemental Figure 1 at <http://www.genetics.org/supplemental/>). Little or no G_1 delay was detectable in comparing the Sic1 mutants to wild type in this protocol, even though *SIC1-OP-PrA* and *SIC1-5P-PrA* strains exhibited a clear increase in G_1 population in asynchronous flow-cytometry profiles compared to wild type (Figure 4). We speculate that this may be due to the protracted G_1 arrest due to α -factor.

Constitutive nuclear localization of Sic1 phosphorylation-site mutants: Sic1 is localized to the nucleus during a brief period between mitosis and G_1/S (NASH *et al.* 2001). This period corresponds roughly to the time

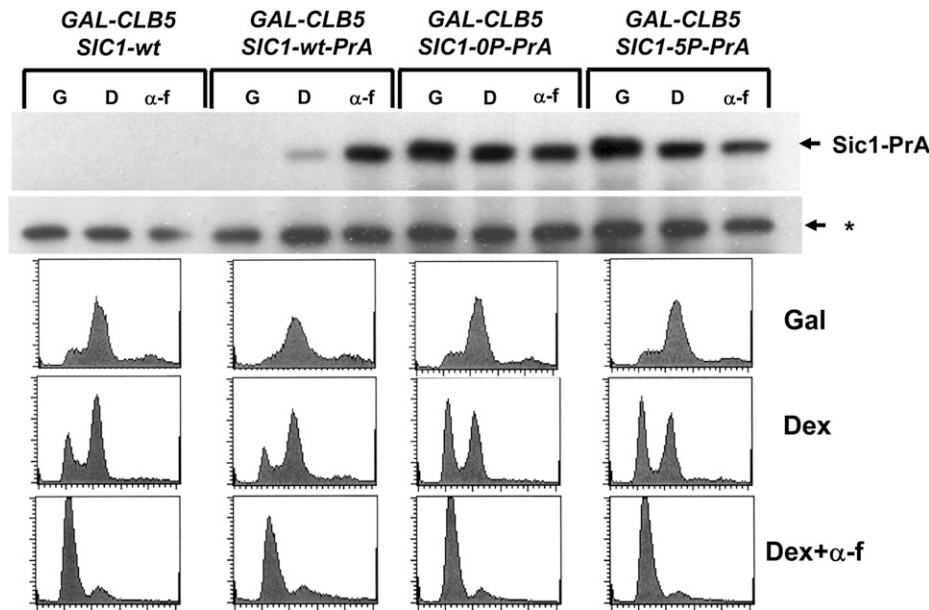


FIGURE 4.—Sic1-0P and Sic1-5P fail to exhibit regulated changes in abundance displayed by wild-type Sic1. Strains carrying *SIC1-wt*, *SIC1-0P*, or *SIC1-5P*, all fused to a C-terminal protein A tag and expressed from the endogenous locus and also carrying a *GALS-CLB5* cassette, were grown in galactose medium to overexpress Clb5 (G), shifted to glucose medium for 2.5 hr to shut off *GALS-CLB5* (D), or to glucose medium plus α-factor (α-f) for 2.5 hr to arrest cells in G₁. (Top) Western blots to detect Sic1-PrA or a control cross-reacting band (*) as loading control. Flow-cytometry profiles for the samples are presented below.

when Sic1 is present in the cell, but Sic1 nuclear localization could still be regulated independently of proteolysis. Since the B-type cyclins regulated by Sic1 have predominantly nuclear localization, the lack of cell cycle arrest by stabilized Sic1 could be due to Sic1 leaving the nucleus even if undegraded. To test this, we fused wild-type Sic1, Sic1-0P, and Sic1-5P to GFP, expressed from

the endogenous locus, and analyzed these strains by time-lapse fluorescence microscopy.

Wild-type Sic1-GFP appeared at about the same time in mother and daughter nuclei (Figure 6), presumably as mitotic exit initiated, and then was detectable for only $\sim 11 \pm 6$ min in mother-cell nuclei. Daughter cells, with a longer G₁, exhibited an $\sim 30 \pm 15$ min period of Sic1

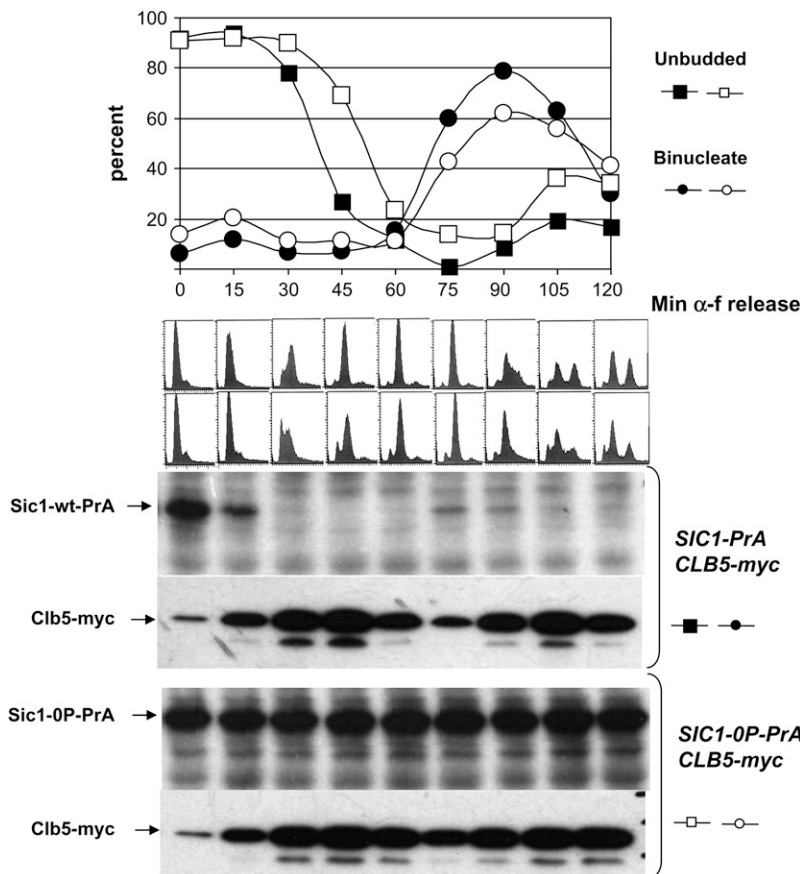


FIGURE 5.—Sic1-0P is stable throughout the cell cycle. *SIC1-wt-PrA* or *SIC1-0P-PrA* *GALS-CLB5 myc-CLB5* strains pregrown in galactose medium were switched to glucose medium plus α-factor (α-f) to arrest cells in G₁, as in Figure 4. After a 2.5 hr arrest, cells were centrifuged, washed twice to remove α-factor, and reinoculated into fresh glucose medium. Time points were taken every 15 min. (Bottom) Western blot analysis of Sic1-PrA and myc-Clb5. The proportions of unbudded and binucleate cells were determined microscopically (top), and the approximate period of DNA replication (between 15 and 45 min after release) was determined by FACS (middle).

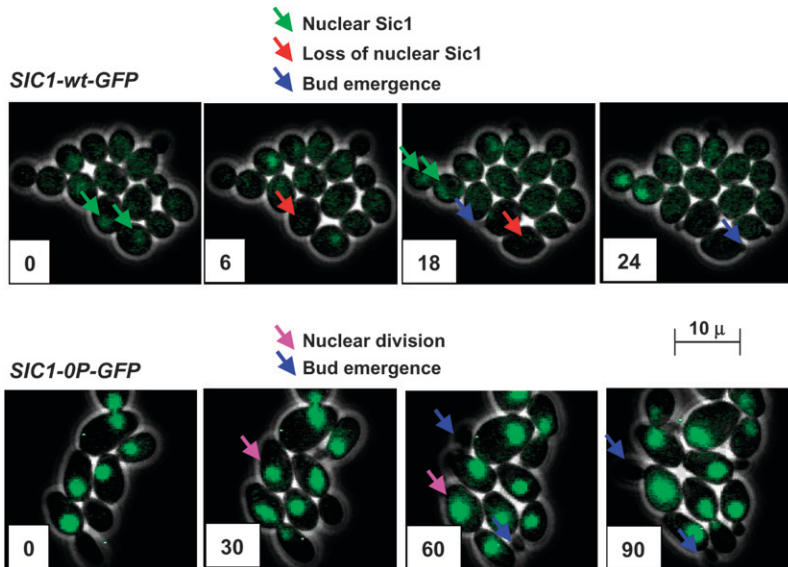


FIGURE 6.—Nuclear Sic1-wt-GFP is detected only during M/G₁; Sic1-OP-GFP is nuclear throughout the cell cycle. Cells containing *S1C1-wt-GFP* or *S1C1-OP-GFP*, expressed from the endogenous locus were analyzed by time-lapse fluorescence microscopy, with illumination every 3 min. Selected frames from movies are shown to illustrate Sic1-wt-GFP appearing as a nuclear signal in large-budded cells (top, green arrows) and then disappearing rapidly from the mother-cell nucleus and more slowly from the daughter-cell nucleus (top, red arrows). Sic1-wt-GFP disappearance occurs shortly before bud emergence (top, blue arrows). Sic1-OP-GFP was nuclear throughout the cell cycle, including before and after bud emergence (bottom, blue arrows) and before and after nuclear division (pink arrows). The disappearance of Sic1-OP-GFP from nuclei was never observed. In both series, for clarity, only some events are labeled with arrows.

nuclear residence. In both mothers and daughters, budding followed loss of the nuclear Sic1 signal by ~10–15 min (Figure 6; our unpublished data).

In contrast, Sic1-OP-GFP was nuclear throughout the cell cycle, including throughout nuclear division and bud emergence (Figure 6). Sic1-5P-GFP gave essentially the same results as Sic1-OP-GFP (supplemental Figure 2 at <http://www.genetics.org/supplemental/>).

Single-cell analysis of the consequences of blocking Sic1 phosphorylation: We characterized the cell cycle of *S1C1-OP* cells by quantitative time-lapse fluorescence microscopy using *CLN2pr-GFP*, encoding unstable GFP under control of the endogenous *CLN2* promoter (MATEUS and AVERY 2000; BEAN *et al.* 2006). *S1C1-OP* cells had *CLN2pr-GFP* peaks of greater intensity than wild type, and the interval between bud emergence and the *CLN2-GFP* peak intensity was significantly increased in the mutant (Figure 7; Table 1). This may reflect a less-efficient shutoff of *CLN2* expression by mitotic Clb activity (AMON *et al.* 1993), due to Clb inhibition by stable Sic1.

The ratio of bud length to width was significantly higher in *S1C1-OP* cells than in wild type (Table 1). This could be due to a longer period of polarized bud growth caused by excessive Cln1/2 and/or reduced mitotic Clb activity (LEW and REED 1993). Interestingly, this ratio was much more variable in *S1C1-OP* cells than in wild-type (standardized variance ratio of 17, $P < 0.001$), suggesting that this effect varied significantly in different cells in the *S1C1-OP* population. Overall, these data suggest that *S1C1-OP* preferentially elongates a period of the cell cycle between budding and transcriptional activation of *CLN2* and the later shutoff of *CLN2* and switch to an isotropic bud growth pattern, both of which are dependent on activity of mitotic B-type cyclins (AMON *et al.* 1993; LEW and REED 1993).

We also carried out movies with wild-type and *S1C1-OP* strains containing *MCM2-GFP* (data not shown). The

Mcm complex enters the nucleus upon mitotic exit and then undergoes regulated nuclear export, dependent on Clb kinase activation (LABIB *et al.* 1999). The period of Mcm2-GFP nuclear residence was significantly elongated in the *S1C1-OP* cells (this period was scored at 57 ± 21 and 45 ± 20 min for wild-type daughters and mothers; for *S1C1-OP*, the period was scored at 77 ± 15 and 62 ± 12 min for daughters and mothers). The period between bud emergence and Mcm2-GFP nuclear exit was greatly elongated in the *S1C1-OP* cells (11 ± 11 and 14 ± 15 min for wild-type daughters and mothers, and 46 ± 13 and 37 ± 13 min for *S1C1-OP* daughters and mothers).

Since activation of G₁ cyclins, including *CLN1* and *CLN2*, drives bud emergence (MOFFAT and ANDREWS 2004), while Clb kinase activation is required for Mcm complex nuclear exit (LABIB *et al.* 1999), these data on timing of Mcm2-GFP nuclear exit support an increased delay between SBF-dependent *CLN2* transcriptional activation and the later activation of Clb kinases in *S1C1-OP* cells, consistent with conclusions reached using *CLN2pr-GFP* time-lapse fluorescence microscopy.

The overall cell division cycle measured by the interval between successive mother-cell bud emergences was longer in the *S1C1-OP* mutant, and the mutant cells were larger (Figure 7, Table 1), probably because of the longer average cell cycle time. Separate movies using wild-type or *S1C1-OP* strains expressing Myo1-GFP as a bud neck/cytokinesis marker (BI *et al.* 1998) indicate that the longer cell cycle in *S1C1-OP* strains is due to a preferential elongation of the part of the cell cycle between budding and cytokinesis; the unbudded period is significantly shorter in *S1C1-OP* cells than in wild-type cells (Table 2). Most likely the unbudded period is shortened because the delay in the budded period results in large progeny (Table 1). Such large cells may escape size control over Start (HARTWELL and UNGER 1977) and therefore bud soon after division.

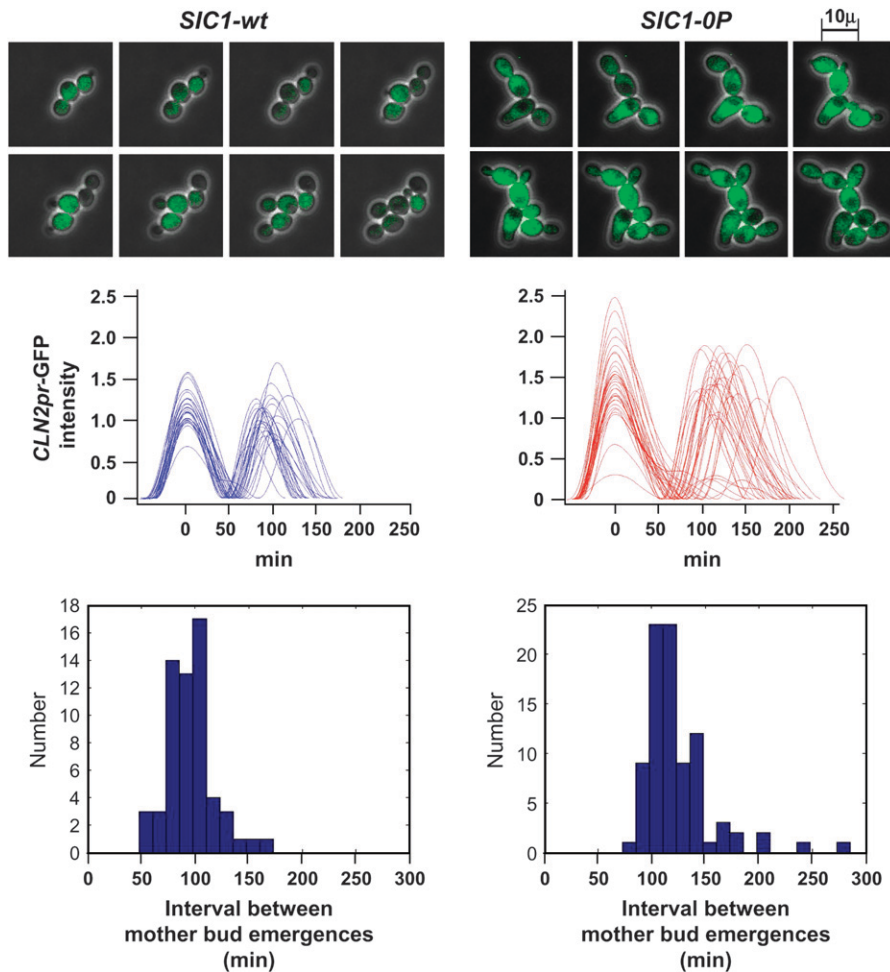


FIGURE 7.—Analysis of the cell cycle in *SIC1-OP* cells by quantitative time-lapse fluorescence microscopy using a *CLN2pr-GFP* marker. Time-lapse fluorescence microscopy on *SIC1-wt* or *SIC1-OP* cells also containing *CLN2pr-GFP* (unstable GFP under control of the *CLN2* promoter) (MATEUS and AVERY 2000; BEAN *et al.* 2006) was carried out as described (BEAN *et al.* 2006) with 3-min resolution. (Top) Composite phase/fluorescence images from representative movies are presented [images taken every 15 min, starting 60 min (*SIC1-wt*) or 101 min (*SIC1-OP*) after plating]. The images were collected in parallel and processed identically. Background fluorescence corresponding to autofluorescence in unlabeled cells was subtracted. Graphs (middle) present data extracted automatically from all data collected (annotated files for microcolonies developed over 9 hr of exponential growth from seven mutant and six wild-type founder cells). The traces (left) represent the smoothed and background-subtracted *CLN2pr-GFP* intensity profiles for all cases where the peak finder found two successive peaks on a common scale for wild type and *SIC1-OP*. The histogram (bottom) indicates the distribution of times between successive mother-cell buddings, measuring the mother-cell cycle time.

In addition to the significant increase in average division time caused by *SIC1-OP* (Table 1), there were a number of outliers with highly increased division times in the *SIC1-OP* strain (Figure 7, bottom right); delays of this magnitude are much less frequent in wild type.

Sic1 stabilization makes major B-type cyclins essential: The simplest model to account for viability of *SIC1-OP* cells, given that Sic1-OP is an effective inhibitor (Figure 3), is that Clb cyclins accumulate to a sufficiently high level to titrate out available Sic1-OP. This idea leads to the prediction that reduction of *CLB* gene dosage should result in *SIC1-OP* lethality.

The S-phase B-type cyclin Clb5 is a major target of Sic1 (SCHWOB *et al.* 1994). *CLB5* deletion was nearly lethal in tetrad analysis when combined with *SIC1-OP* (Figure 8). This lethality was partially suppressed by simultaneous deletion of *SWI5*. Tetrad analysis with *SIC1* mutants containing various combinations of phosphorylation-site mutations (Figure 8) yielded the interesting result that some *SIC1* mutants retaining phosphorylation sites gave a more profound block to viability in the absence of *clb5* than did *SIC1-OP*, in some cases resulting in complete inviability of even *swi5* segregants. This suggests the hypothesis that stable Sic1 retaining some phosphorylation sites might be a more potent *in vivo* inhibitor than

fully unphosphorylatable Sic1. Despite this, all *SIC1* mutants could be recovered in tetrad analysis in a *CLB5 SWI5* background with high viability.

We could recover viable *SWI5 clb5 SIC1-OP* segregants that also contained *GALS-CLB5* on galactose medium (*GALS-CLB5* on). These strains were essentially inviable on glucose medium (Figure 8B). Upon shifting to glucose medium for 3 hr to shut off *GALS-CLB5*, *clb5 SIC1-OP GALS-CLB5* strains exhibited an accumulation of G₁ cells much higher than that observed with *clb5 SIC1-wt GALS-CLB5* controls, suggesting that initiation of replication was delayed by *SIC1-OP* in this background (supplemental Figure 3 at <http://www.genetics.org/supplemental/>). Most of the cells had long buds, reminiscent of cells lacking B-type cyclin activity (SCHWOB *et al.* 1994). The *clb5 SIC1-OP* block was somewhat leaky (as reflected by sporadic recovery of weakly viable *clb5 SIC1-OP* segregants in tetrad analysis) (Figure 8A); therefore, we did not characterize the *GALS-CLB5* shutoff phenotype in great detail.

Four B-type cyclins, *CLB1–4*, act to promote mitosis. The Clb1/Clb2 and Clb3/Clb4 pairs are close sequence homologs (FITCH *et al.* 1992). Clb2 and Clb3 are present in higher protein copy number than Clb1 and Clb4 (CROSS *et al.* 2002). We tested combinations of cyclin deletions

TABLE 1
***CLN2pr-GFP* expression and other statistics from quantitative time-lapse fluorescence microscopy**

Measure	<i>SIC1</i> genotype	Mean \pm SD (<i>n</i>)	Wild type <i>vs.</i> <i>SIC1-OP</i> (<i>t</i> -test)
<i>CLN2pr-GFP</i> peak amplitude (AU)	Wild type	1.04 \pm 0.23 (57)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	1.46 \pm 0.40 (65)	
<i>CLN2pr-GFP</i> peak width (in minutes)	Wild type	47 \pm 5.6 (57)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	54 \pm 5.6 (70)	
Budding to <i>CLN2pr-GFP</i> peak (in minutes)	Wild type	21 \pm 8 (88)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	27 \pm 6 (89)	
Cell size at budding (pixels)	Wild type	1983 \pm 311 (104)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	2914 \pm 587 (127)	
Mother-cell cycle time (in minutes)	Wild type	95 \pm 19 (59)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	121 \pm 25 (85)	
Bud long axis/short axis length ratio (60 min after budding)	Wild type	1.1 \pm 0.06 (50)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	1.7 \pm 0.389 (81)	

Data were obtained as described in BEAN *et al.* (2006). Nine-hour recordings (images every 3 min), starting with seven mutant and six wild-type founder cells containing *CLN2pr-GFP* were made, images were automatically segmented and assigned background-subtracted GFP signal values for each cell body, and bud emergence timing and mother–daughter relationships were assigned using the graphical user interface (BEAN *et al.* 2006). Computations are described in MATERIALS AND METHODS. Bud dimensions were determined using ImagePro-Plus on randomly selected cells 60 min after bud emergence. AU, arbitrary units.

with *SIC1-OP* by tetrad analysis, scoring the viability of segregants of various genotypes. *clb2 SIC1-OP* and *clb3 clb4 SIC1-OP* segregants were completely inviable in tetrad analysis. *clb4 SIC1-OP* segregants were moderately slow growing; *clb3 SIC1-OP* segregants formed extremely slow-growing microcolonies. These results, combined with the lethality of *clb5 SIC1-OP* segregants, suggest that *SIC1-OP* imposes a requirement for a critical level of B-type cyclin without requirements as to Clb sequence class.

We could recover viable *clb2 SIC1-OP GALS-CLB2* strains by tetrad analysis on galactose medium (*GALS-CLB2* on). These strains were tightly inviable when transferred to glucose medium (*GALS-CLB2* off) (Figure 9). We determined the lethal phenotype of *clb2 SIC1-OP* by transferring *clb2 SIC1-OP GALS-CLB2* strains from galactose to glucose medium for 3 hr. The cells exhibited an arrest with 2C DNA content, indicating completion of DNA replication but failure to complete mitosis (Figure 9); most of the cells had long buds and a single nucleus

TABLE 2
Preferential elongation of the budded part of the cell cycle in *SIC1-OP* cells

Measure	<i>SIC1</i> genotype	Mean \pm SD (<i>n</i>)	Wild type <i>vs.</i> <i>SIC1-OP</i> (<i>t</i> -test)
Unbudded period, daughters	Wild type	49 \pm 27 (13)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	16 \pm 6 (29)	
Budded period, daughters	Wild type	71 \pm 17 (10)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	119 \pm 40 (14)	
Cell cycle, daughters	Wild type	119 \pm 28 (10)	<i>P</i> < 0.25
	<i>SIC1-OP</i>	134 \pm 41 (14)	
Unbudded period, mothers	Wild type	25 \pm 8 (15)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	14 \pm 9 (27)	
Budded period, mothers	Wild type	65 \pm 17 (10)	<i>P</i> < 0.001
	<i>SIC1-OP</i>	103 \pm 19 (14)	
Cell cycle, mothers	Wild type	90 \pm 16 (10)	<i>P</i> < 0.005
	<i>SIC1-OP</i>	116 \pm 18 (10)	

MYO1-GFP cells, either *SIC1-wt* or *SIC1-OP*, were analyzed by time-lapse microscopy, and the times of budding and Myo1 ring formation and cytokinesis, as marked by Myo1 ring closure and disappearance (Bi *et al.* 1998), were scored manually. The unbudded period was defined as the time from cytokinesis to budding, and the budded period as the time from budding to cytokinesis. The total cell cycle was the time from one cytokinesis to the next. Data for mothers and daughters were analyzed separately.

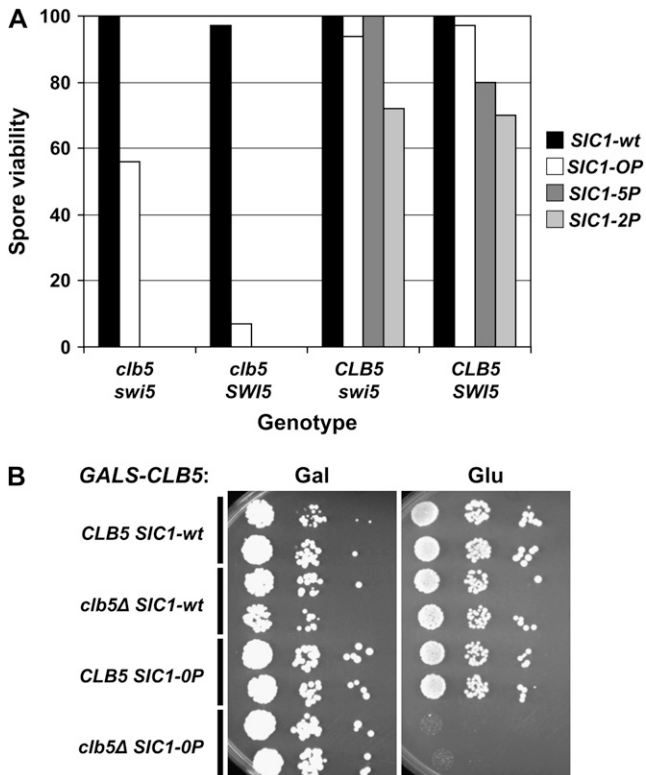


FIGURE 8.—Phosphorylation-site mutations in *SIC1* result in lethality in the absence of *CLB5*. (A) Tetrad analysis was performed on diploids of genotypes *swi5::kanMX/+ clb5::URA3/+*, *sic1::HIS3/SIC1-wt*, *sic1::HIS3/SIC1-OP*, *sic1::HIS3/SIC1-5P*, or *sic1::HIS3/SIC1-2P*. *SIC1-5P*, T2A, T5A, T33A, S76A; *SIC1-2P*, T33A, S69A, S76A, T80A, S174A, NS T192A. Spore viability for various genotypes was assessed assuming 2:2 segregation for all markers. Rare viable segregants of the genotype *clb5 SWI5 SIC1-OP* were small, slow-growing colonies. (B) *GALS-CLB5 SIC1-OP clb5::URA3* strains and controls were constructed by tetrad analysis on galactose medium. Serial dilutions on galactose medium (Gal; *GALS-CLB5* on) and glucose medium (Glu; *GALS-CLB5* off) were incubated for 3 days at 30°.

(Figure 10). This phenotype resembles that of cells lacking the mitotic cyclins *CLB1–4* (FITCH *et al.* 1992).

clb2 clb3 clb4 SIC1-wt GAL-CLB2 strains arrest with 2C DNA upon turnoff of *GAL-CLB2* (FITCH *et al.* 1992) (Figure 9), since they complete replication due to Clb5, -6 activity (SCHWOB *et al.* 1994), but then fail to complete mitosis due to deficiency of mitotic cyclins Clb1–4. Strikingly, *clb2 clb3 clb4 SIC1-OP GAL-CLB2* strains arrested with 1C DNA (Figure 9), indicating that Clb5 and Clb6 are insufficient for driving replication in the *clb2 clb3 clb4 SIC1-OP* background. This 1C arrest, with long buds (our unpublished data), resembles that of cells lacking all B-type cyclins (SCHWOB *et al.* 1994).

clb3 clb4 SIC1-OP GAL-CLB2 strains showed inviability on glucose medium; *clb3 clb4 SIC1-wt* strains, in contrast, are viable (FITCH *et al.* 1992). *clb3 clb4 SIC1-OP GAL-CLB2* strains transferred to glucose medium for 3 hr showed a predominantly 1C arrest (Figure 9), suggesting that, in this background, Sic1-OP effectively inhibits

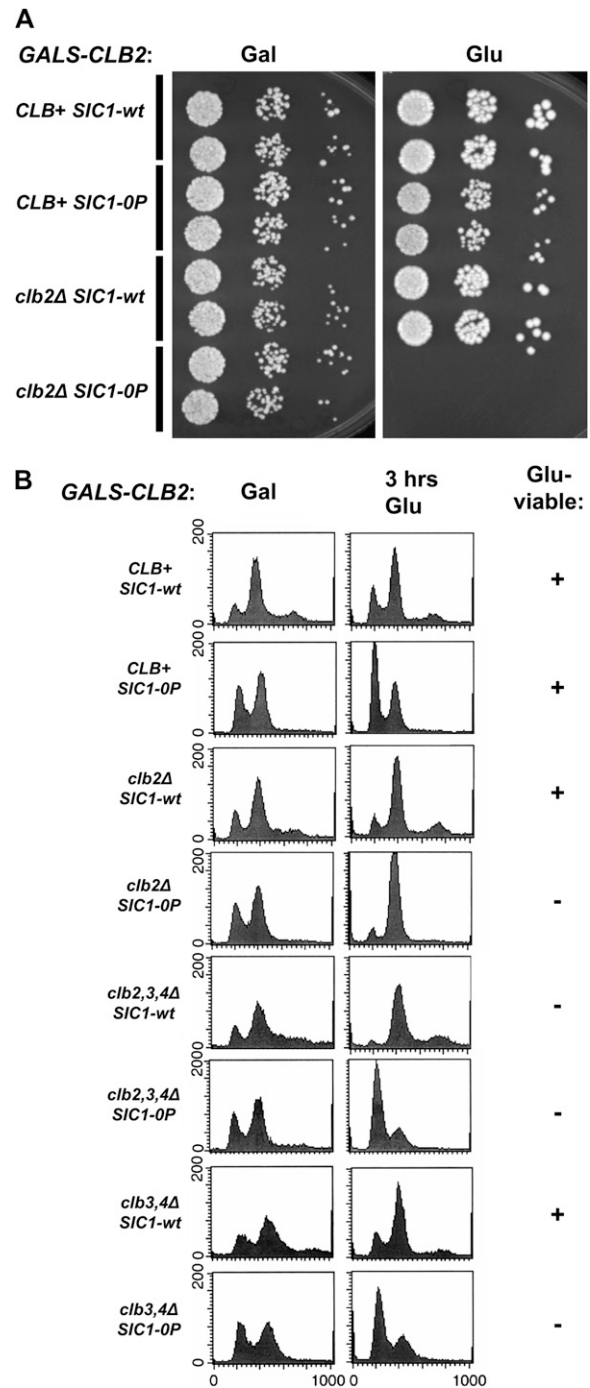


FIGURE 9.—Requirements for mitotic *CLB2–4* in a *SIC1-OP* background. (A) *GALS-CLB2 SIC1-OP clb2::LEU2* strains and controls were constructed by tetrad analysis on galactose medium. Serial dilutions on galactose medium (Gal; *GALS-CLB* on) and glucose medium (Glu; *GALS-CLB* off) were incubated for 3 days at 30°. (B) Flow-cytometry analysis of *GALS-CLB2* strains, either *SIC1-wt* or *SIC1-OP*, containing the indicated additional *CLB* gene deletions. Strains were grown in galactose medium (Gal) or transferred to glucose medium (Glu) for 3 hr. (Right) Viability of the indicated genotype on glucose medium (*GALS-CLB2* off) as determined by a replica-plating patch assay.

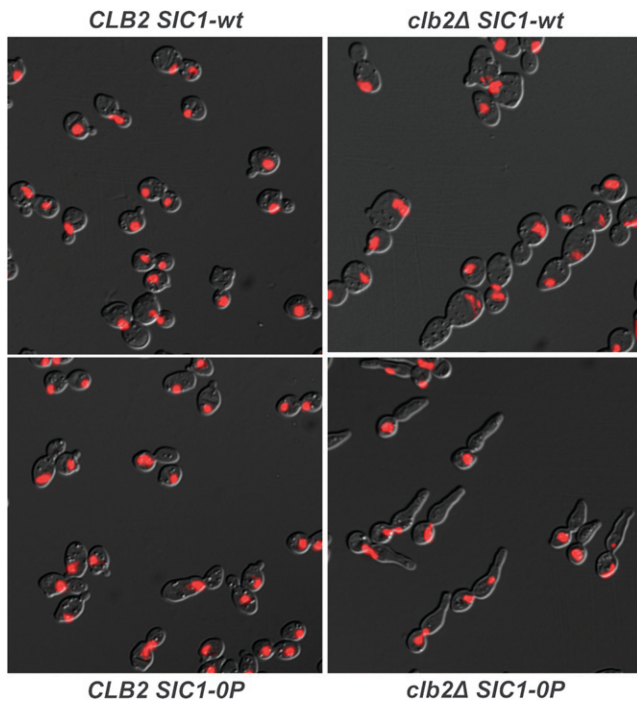


FIGURE 10.—Lethal phenotype of *clb2 SIC1-0P* cells. Cells of the indicated genotype, also containing a *GALS-CLB2* cassette, were grown in galactose medium to log phase and transferred to glucose medium for 3 hr. Cells were fixed in ethanol and digested with RNase and protease and nuclear DNA was stained with propidium iodide; samples were examined by DIC and fluorescence microscopy, and composite images were generated. All exposure settings were identical for these images.

the S-phase cyclins Clb5, -6 as well as the remaining mitotic B-type cyclins Clb1, -2, resulting overall in Clb-dependent kinase activity insufficient for driving DNA replication.

Viability of cells expressing Sic1 lacking ubiquitin-accepting lysines: Removal of six lysines from the N-terminal half of Sic1 significantly inhibits Sic1 ubiquitination and turnover; these lysines may be the primary acceptor sites for SCF-Cdc4-dependent ubiquitination (PETROSKI and DESHAIES 2003a). We replaced *SIC1* with *SIC1-K0N* (with these six lysines replaced by arginines) under control of the endogenous promoter in a *swi5* background, as described above for *SIC1-0P* (see Figure 1). Subsequent tetrad analysis employing essentially the assay described in Figure 8 revealed ~100% viability of *SIC1-K0N SWI5* cells; these cells exhibited a G₁ delay by flow cytometry (supplemental Figure 5 at <http://www.genetics.org/supplemental/>). Deletion of *CLB5* sharply reduced viability of *SIC1-K0N* segregants, essentially as observed with *SIC1-0P* (~17% compared to ~100% for *SIC1-wt clb5* strains and ~7% for *SIC1-0P clb5* segregants; see Figure 8), and the rare viable *SIC1-K0N clb5* segregants were notably slow growing in tetrad analysis. These results thus recapitulate the effects of removing Sic1 phosphorylation sites by removing instead ubiquitin-accepting lysine residues in Sic1 but leaving the phosphorylation sites intact.

In an α -factor block-release experiment using cells expressing endogenous levels of Sic1-K0N-PrA compared to Sic1-wt-PrA, little difference was observed in the time of DNA replication between the two strains, and replication occurred after Sic1-wt-PrA was degraded. In contrast, most cells had completed DNA replication in the presence of high levels of Sic1-K0N-PrA, although partial reduction in Sic1-K0N-PrA levels were detectable later in the cell cycle (Figure 11). The partial, slow residual ubiquitination and degradation of Sic1-K0N presumably operates through inefficiently used C-terminal lysine-ubiquitin acceptors (PETROSKI and DESHAIES 2003a).

Sic1-K0N-GFP (supplemental Figure 6 at <http://www.genetics.org/supplemental/>) exhibited intermediate behavior between Sic1-wt and Sic1-0P (Figure 6) or Sic1-5P (supplemental Figure 2 at <http://www.genetics.org/supplemental/>). Sic1 appeared at the time of mitotic exit in mother and daughter nuclei similarly to wild type, but the Sic1 nuclear signal then decayed slowly compared to wild type. As noted above, the period of Sic1-wt-GFP nuclear residence was $\sim 11 \pm 6$ min in mother-cell nuclei and $\sim 30 \pm 15$ min in daughter-cell nuclei. In contrast, Sic1-K0N-GFP nuclear residence was $\sim 35 \pm 27$ min in mother-cell nuclei and $\sim 55 \pm 26$ min in daughter-cell nuclei. The Sic1-K0N-GFP nuclear signal almost always persisted until well after bud emergence ($\sim +25 \pm 25$ min), while the Sic1-wt-GFP signal always disappeared before bud emergence ($\sim -12 \pm 8$ min). Ultimate loss of Sic1-K0N-GFP nuclear signal is likely due at least in part to partial degradation of Sic1-K0N (Figure 11), although a contribution of regulated nuclear export of Sic1-K0N-GFP cannot be ruled out. In any case, this behavior of Sic1-K0N-GFP in single cells strongly suggests persistence of significant levels of Sic1-K0N until after DNA replication, because of the known correlation between bud emergence and DNA replication, as confirmed, for example, in the α -factor block-release experiment in Figure 11.

Thus, overall, results with Sic1-K0N suggest that even phosphorylatable Sic1 that fails to be degraded is unable to block DNA replication.

DISCUSSION

Sic1 phosphorylation is not essential: It was proposed that Sic1 phosphorylation is essential for the G₁/S transition (SCHWOB *et al.* 1994; VERMA *et al.* 1997a). Our data present a serious challenge to this hypothesis: *SIC1-0P* cells are viable despite stability and constitutive nuclear localization of a high level of functional Sic1. Replication is delayed in *SIC1-0P* cells, probably due to delayed activation of Clb kinases.

How can we reconcile our findings with previous results suggesting the essentiality of Sic1 phosphorylation and degradation? The finding (SCHWOB *et al.* 1994) that *sic1 cdc4* mutants fail to arrest in G₁, unlike *SIC1 cdc4*

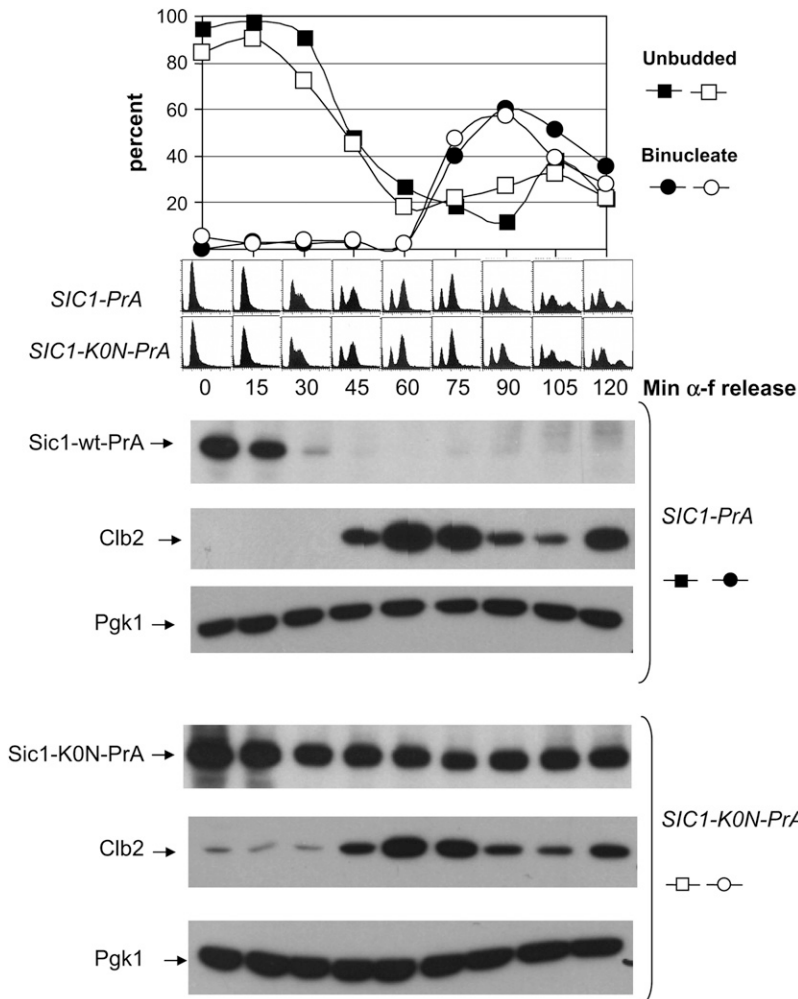


FIGURE 11.—Sic1-K0N is relatively stable throughout the cell cycle. *SIC1-wt-PrA* or *SIC1-K0N-PrA* strains were grown to log phase in glucose medium and then α -factor (α -f) was added to arrest cells in G₁, as in Figure 4. After a 2.5 hr arrest, cells were centrifuged and washed twice to remove α -factor and reinoculated into fresh glucose medium. Time points were taken every 15 min. (Bottom) Western blot analysis of Sic1-PrA, Clb2, and Pgk1 as a loading control. The proportions of unbudded and binucleate cells were determined microscopically, and the approximate period of DNA replication (between 15 and 45 min after release) by flow cytometry (middle panels).

mutants, strongly suggests that undegraded Sic1 at endogenous levels is sufficient for blocking replication, at least in a *cdc4* mutant background. One possible explanation of the discrepancy may be that fully phosphorylated Sic1, such as accumulates in a *cdc4* block, is a more effective inhibitor *in vivo* than unphosphorylated Sic1. *SIC1* mutants containing some phosphorylation sites intact are more deleterious in tetrad analysis than *SIC1-0P* (Figure 8), which could be consistent with this explanation. However, our data show that Sic1-0P was as effective as wild type at Clb5-Cdc28 binding and inhibition *in vitro* (Figure 3), confirming previous results (VERMA *et al.* 1997a; NASH *et al.* 2001). Sic1 lacking the primary ubiquitin-acceptor lysines is phosphorylated, but nevertheless is significantly stabilized. Therefore, as an additional test of the idea that Sic1-0P might be a partially defective *in vivo* inhibitor due to lack of phosphorylation sites, we tested whether Sic1 lacking these lysines (Sic1-K0N), expressed from the endogenous *SIC1* promoter, would have a more deleterious phenotype than *SIC1-0P*; however, this allele allowed full viability with a G₁ delay phenotype, similar to that observed with *SIC1-0P* (supplemental Figure 5 at <http://www.genetics.org/supplemental/>). This result is consistent with the idea that stable Sic1, even if phosphor-

ylatable, delays but does not block DNA replication, when expressed from the endogenous promoter. A complication in this interpretation is the partial degradation of Sic1-K0N (PETROSKI and DESHAIES 2003a) (Figure 11) and its loss from the nucleus late in the cell cycle (supplemental Figure 6 at <http://www.genetics.org/supplemental/>). Still, the results with Sic1-K0N are supportive of the results with Sic1-0P in suggesting that Sic1 proteolysis is not essential. Sic1-K0N-expressing cells are able to synthesize DNA on schedule in an α -factor block-release protocol while significant levels of Sic1-K0N persist (Figure 11), although some drop in the Sic1-K0N level is notable later in the time course. While *SIC1-K0N CLB5* and *SIC1-0P CLB5* segregants are fully viable, *SIC1-K0N* segregants lacking *clb5* are strongly reduced in viability (see above), as are *SIC1-0P clb5* segregants (Figure 8), suggesting that both these mutations sensitize the cell to lowered levels of B-type cyclins.

Another possibility is that the requirement for Sic1 phosphorylation is somehow restricted to the SCF-Cdc4-deficient background. We found a strong interaction between *cdc4-1* and *SIC1-0P*, such that the double mutants were significantly slowed in growth rate even at permissive temperature (our unpublished data), and this interaction limited our ability to pursue this issue in more

depth. This is because we were concerned that the double mutants might accumulate suppressors or modifiers due to cryptic selection from slow growth, rendering results with them difficult to interpret. *SIC1* is haplo-insufficient in a *cdc4* background for restraining replication (KNAPP *et al.* 1996), suggesting that even if Sic1 is in excess of Clb kinases in this background, this excess is within a factor of 2. The alternative that Sic1 degradation is critical only at higher temperatures is probably not correct since *SIC1-OP* cells are not temperature sensitive for viability and they show no further increase in G₁ accumulation by flow-cytometry analysis upon shift to 37° (data not shown).

Finally, we cannot rule out that the phosphorylation-site mutations (and perhaps the Lys-Arg mutations in the *SIC1-KON* mutant) subtly perturb the structure of Sic1 such that it is a less effective inhibitor: these putative alterations in structure might not even be related to phosphorylation, but rather related to changes in function of the unphosphorylated protein by some of the mutational changes. This hypothetical reduction in efficiency of Sic1 would have to result in minimal or undetectable changes in efficiency of *in vitro* inhibition (Figure 3) (NASH *et al.* 2001; PETROSKI and DESHAIES 2003a) and would also call into question the generally accepted modular structure of Sic1, with the N-terminal regions dedicated to the phosphodegron and only the C-terminal 70 amino acids being involved in Cdk inhibition (HODGE and MENDENHALL 1999; NASH *et al.* 2001). For these reasons, we do not favor the hypothesis that the phosphorylation-site mutations weaken direct Cdk inhibition, although we cannot rule out this hypothesis at this time.

SIC1-del3P (mutated in four phosphorylation sites, T2, T5, T33, and T76) blocked entry into S phase when overexpressed only very moderately (VERMA *et al.* 1997a). We reconstructed this mutant under control of the endogenous promoter (our *SIC1-5P*). Like *SIC1-OP*, *SIC1-5P* encodes a highly stable, constitutively nuclear protein that elongates G₁ but does not result in inviability or in a block to replication in an α -factor block-release experiment (Figure 4; supplemental Figures 1 and 2 at <http://www.genetics.org/supplemental/>).

Lethality of *SIC1-OP* when combined with deletion of the normally nonessential B-type cyclins *CLB2*, *CLB3*, or *CLB5* suggests that unphosphorylatable Sic1 is just below a lethal threshold, so even moderate overexpression of unphosphorylatable Sic1 (VERMA *et al.* 1997a) may block DNA replication. Consistent with this idea, transformation of a *sic1::HIS3* strain with a CEN (low-copy-number) plasmid containing *SIC1-OP* (endogenous promoter) gives notably slow-growing colonies compared to vector or wild-type *SIC1* plasmid (supplemental Figure 4 at <http://www.genetics.org/supplemental/>).

In summary, our data very strongly suggest that Sic1 phosphorylation is not essential. While it is impossible, using Sic1 phosphorylation-site mutants, to rule out the hypothesis that allowing Sic1 phosphorylation but then

preventing its degradation might be essential, the latter situation is essentially an artifact of fully phosphorylated Sic1 encountering an experimentally disrupted Sic1 removal system. Evolutionarily, it appears likely that Sic1 initially evolved as a Cdk inhibitor that later incorporated phosphorylation-dependent degradation, rather than the other way around; thus, *SIC1-OP* is much more likely to be similar to the precursor of the fully evolved system. Therefore, our data emphasize that a fully functional Cdk inhibitory system is compatible with the complete absence of regulated proteolysis or localization of the inhibitor, and we speculate that such a system probably evolved first, before the development of the regulated proteolysis system.

Sic1 phosphorylation provides a Cln-dependent sluice gate to a Sic1 dam restraining B-type cyclin-dependent kinase: We explain viability of *SIC1-OP* cells by proposing that stable Sic1 transiently blocks Clb-Cdk activation, but that ultimately the total level of Clb-Cdk accumulates to above the level of the Sic1 blockade. Since Sic1 is a stably bound stoichiometric inhibitor, early-accumulating Clb-Cdk could effectively titrate Sic1, remaining bound even when later-accumulating Clb-Cdk is fully active. This model accounts for the lethal phenotypes of *SIC1-OP* when it is combined with various *CLB* deletions: normally nonessential *CLB* genes become essential, presumably because there is insufficient residual Clb protein to titrate Sic1-OP (Figure 9). The final phenotype (1C or 2C arrest; Figure 9) presumably reflects some combination of differential replication-promoting *vs.* mitosis-promoting activity of different Clb proteins and the varying contribution of different Clb proteins to total Clb levels attained at various points in the cell cycle (MILLER and CROSS 2001; CROSS *et al.* 2002). This model for titration of stable Sic1 is formally similar to the proposal that cyclin D complexes activated early in the mammalian cell cycle titrate the p27 inhibitor and thus indirectly activate later-accumulating cyclin E and cyclin A complexes (SHERR and ROBERTS 1999).

NASMYTH and HUNT (1993) proposed a “dams and sluices” analogy for Cdk inhibitors. A simple dam blocks water flow until the water level rises above the dam. A sluice gate is a regulatable opening at the base of the dam. A dam with a sluice gate provides all the functionality of a simple dam, blocking downstream water flow until the level becomes high, but can also release all of the upstream accumulated water, independent of its level. Sic1 can be analogized as a dam for accumulated Clb’s and Cln-dependent phosphorylation as opening the sluice gate. Our data reveal the nonlethal, but still significant, flaws in the Sic1-OP system (a dam without a sluice gate): this system demands that Clb-Cdk accumulates to high levels, unlike the wild-type system, accounting for the elongated G₁ period and the genetic requirement for *CLB2*, *CLB3*, and *CLB5* observed in the *SIC1-OP* background.

Rates of production of many different proteins may vary significantly across a cell population (BAR-EVEN

et al. 2006; NEWMAN *et al.* 2006). The Cln-Cdk sluice-gate control of Sic1 levels may buffer the system to variation in rates of accumulation of Sic1 or Clb proteins. Such variation may account for the sporadic occurrence of highly delayed cell cycles in *SIC1-OP* cells (Figure 7).

Cln-Cdk removal of Sic1 is catalytic, and Cln-Cdk is probably in stoichiometric excess to the Sic1 catalytic target (CROSS *et al.* 2002). As a consequence, wild-type cells can cope with at least 10 extra copies of the *SIC1* gene without significant slowing of growth rate (THORNTON and TOCZYSKI 2003; MORIYA *et al.* 2006). This ability is dependent on Cln1 and Cln2, since *cln1 cln2* cells are inviable with only a few extra copies of the *SIC1* gene (TYERS 1996). What sets the peak level of Sic1 in wild-type cells, given that much more could be dealt with expeditiously? The level (~1000 copies/cell) is similar to the subsequent peak levels of Clb5 and then Clb2 (CROSS *et al.* 2002). Therefore, these levels may provide another level of robustness: the relatively low levels of Sic1 ensure that the simple dam mechanism for providing a Clb-Cdk-free period in early G₁ could operate as a backup. Occasional cell cycles with inefficient expression of Cln1 and Cln2 would provide a selection keeping the peak level of Sic1 reasonably tuned to the peak levels of the target Clb cyclins.

Thus the Sic1 proteolytic removal system may have evolved due to the requirement for a transient block to Clb-Cdk activity in G₁, with the additional constraints of robustness to high expression of Sic1 and to low expression of the Clb targets of Sic1 and the Cln antagonists of Sic1 that normally drive its removal.

A recent computational model (CHEN *et al.* 2004) predicts that, in cells expressing unphosphorylatable Sic1 from the endogenous promoter, DNA replication would occur after a very long G₁ period, consisting of more than two wild-type doubling times (K. CHEN, personal communication). Some revision of the model is required to fit the present data, since the elongated G₁ in *SIC1-OP* is significantly less than a normal wild-type doubling time; however, minor alterations, such as doubling the expression level of *CLB5* or halving the expression level of *SIC1*, will largely rescue the overly long G₁ phenotype predicted by the model (our unpublished data). Inclusion of Clb3 in the model, at appropriate expression levels (CROSS *et al.* 2002), might be an effective and realistic revision, since accumulation of Clb3 in mid-cell cycle would help to titrate stable Sic1, accounting for the observed dependence on Clb3 for viability in the *SIC1-OP* background.

Precise gene replacement for precise answers? Analogously to the value of null alleles for determining the function of a protein in an otherwise wild-type system, the role of a specific sequence within a protein may be best analyzed with a precise gene replacement of the wild type with a version mutated in the specific sequence, with no other changes in promoter or flanking sequences. Here, this strategy has provided data strongly suggesting that contradictory to prior expectations, Sic1

phosphorylation is not essential for removing a lethal block to the G₁/S transition; rather, it performs the nonessential but important role of increasing cell cycle precision and robustness.

Thanks go to A. Amon, R. Deshaies, M. Tyers, and R. Verma for providing plasmids. Thanks also go to J. Bloom and C. Lookingbill for strains and to K. Chen for communicating unpublished modeling results. This work was supported by National Institutes of Health grant PHS GM047238 to F.R.C.

LITERATURE CITED

- AMON, A., M. TYERS, B. FUTCHER and K. NASMYTH, 1993 Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell* **74**: 993–1007.
- BAR-EVEN, A., J. PAULSSON, N. MAHESHRI, M. CARMÍ, E. O'SHEA *et al.*, 2006 Noise in protein expression scales with natural protein abundance. *Nat. Genet.* **38**: 636–643.
- BEAN, J. M., E. D. SIGGIA and F. R. CROSS, 2006 Coherence and timing of cell cycle Start examined at single-cell resolution. *Mol. Cell* **21**: 3–14.
- BI, E., P. MADDOX, D. J. LEW, E. D. SALMON, J. N. McMILLAN *et al.*, 1998 Involvement of an actomyosin contractile ring in *Saccharomyces cerevisiae* cytokinesis. *J. Cell Biol.* **142**: 1301–1312.
- CHEN, K. C., L. CALZONE, A. CSIKASZ-NAGY, F. R. CROSS, B. NOVAK *et al.*, 2004 Integrative analysis of cell cycle control in budding yeast. *Mol. Biol. Cell* **15**: 3841–3862.
- CROSS, F. R., 2003 Two redundant oscillatory mechanisms in the yeast cell cycle. *Dev. Cell* **4**: 741–752.
- CROSS, F. R., V. ARCHAMBAULT, M. MILLER and M. KLOVSTAD, 2002 Testing a mathematical model of the yeast cell cycle. *Mol. Biol. Cell* **13**: 52–70.
- DIRICK, L., T. BOHM and K. NASMYTH, 1995 Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. *EMBO J.* **14**: 4803–4813.
- EPSTEIN, C. B., and F. R. CROSS, 1992 CLB5: a novel B cyclin from budding yeast with a role in S phase. *Genes Dev.* **6**: 1695–1706.
- FITCH, I., C. DAHMANN, U. SURANA, A. AMON, K. NASMYTH *et al.*, 1992 Characterization of four B-type cyclin genes of the budding yeast *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **3**: 805–818.
- HARTWELL, L. H., and M. W. UNGER, 1977 Unequal division in *Saccharomyces cerevisiae* and its implications for the control of cell division. *J. Cell Biol.* **75**: 422–435.
- HODGE, A., and M. MENDENHALL, 1999 The cyclin-dependent kinase inhibitory domain of the yeast Sic1 protein is contained within the C-terminal 70 amino acids. *Mol. Gen. Genet.* **262**: 55–64.
- JACOBSON, M. D., S. GRAY, M. YUSTE-ROJAS and F. R. CROSS, 2000 Testing cyclin specificity in the exit from mitosis. *Mol. Cell Biol.* **20**: 4483–4493.
- KNAPP, D., L. BHOITE, D. J. STILLMAN and K. NASMYTH, 1996 The transcription factor Swi5 regulates expression of the cyclin kinase inhibitor p40SIC1. *Mol. Cell Biol.* **16**: 5701–5707.
- LABIB, K., J. F. DIFFLEY and S. E. KEARSEY, 1999 G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. *Nat. Cell Biol.* **1**: 415–422.
- LEW, D. J., and S. I. REED, 1993 Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *J. Cell Biol.* **120**: 1305–1320.
- MALEK, N. P., H. SUNDBERG, S. MCGREW, K. NAKAYAMA, T. R. KYRIAKIDES *et al.*, 2001 A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. *Nature* **413**: 323–327.
- MATEUS, C., and S. V. AVERY, 2000 Destabilized green fluorescent protein for monitoring dynamic changes in yeast gene expression with flow cytometry. *Yeast* **16**: 1313–1323.
- MENDENHALL, M. D., 1993 An inhibitor of p34CDC28 protein kinase activity from *Saccharomyces cerevisiae*. *Science* **259**: 216–219.
- MILLER, M. E., and F. R. CROSS, 2001 Cyclin specificity: How many wheels do you need on a unicycle? *J. Cell Sci.* **114**: 1811–1820.
- MOFFAT, J., and B. ANDREWS, 2004 Late-G1 cyclin-CDK activity is essential for control of cell morphogenesis in budding yeast. *Nat. Cell Biol.* **6**: 59–66.

- MORGAN, D. O., and J. M. ROBERTS, 2002 Oscillation sensation. *Nature* **418**: 495–496.
- MORIYA, H., Y. SHIMIZU-YOSHIDA and H. KITANO, 2006 In vivo robustness analysis of cell division cycle genes in *Saccharomyces cerevisiae*. *PLoS Genet.* **2**: e111.
- MUMBERG, D., R. MULLER and M. FUNK, 1995 Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156**: 119–122.
- NASH, P., X. TANG, S. ORLICKY, Q. CHEN, F. B. GERTLER *et al.*, 2001 Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. *Nature* **414**: 514–521.
- NASMYTH, K., 1996 At the heart of the budding yeast cell cycle. *Trends Genet.* **12**: 405–412.
- NASMYTH, K., and T. HUNT, 1993 Cell cycle: dams and sluices. *Nature* **366**: 634–635.
- NEWMAN, J. R., S. GHAEMMAGHAMI, J. IHMELS, D. K. BRESLOW, M. NOBLE *et al.*, 2006 Single-cell proteomic analysis of *S. cerevisiae* reveals the architecture of biological noise. *Nature* **441**: 840–846.
- NISHIZAWA, M., M. KAWASUMI, M. FUJINO and A. TOHE, 1998 Phosphorylation of sic1, a cyclin-dependent kinase (Cdk) inhibitor, by Cdk including Pho85 kinase is required for its prompt degradation. *Mol. Biol. Cell* **9**: 2393–2405.
- ORLICKY, S., X. TANG, A. WILLEMS, M. TYERS and F. SICHERI, 2003 Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. *Cell* **112**: 243–256.
- PETROSKI, M. D., and R. J. DESHAIES, 2003a Context of multiubiquitin chain attachment influences the rate of Sic1 degradation. *Mol. Cell* **11**: 1435–1444.
- PETROSKI, M. D., and R. J. DESHAIES, 2003b Redundant degrons ensure the rapid destruction of Sic1 at the G1/S transition of the budding yeast cell cycle. *Cell Cycle* **2**: 410–411.
- SCHWAB, M., A. S. LUTUM and W. SEUFERT, 1997 Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* **90**: 683–693.
- SCHWOB, E., T. BOHM, M. D. MENDENHALL and K. NASMYTH, 1994 The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. *Cell* **79**: 233–244.
- SHERR, C. J., and J. M. ROBERTS, 1999 CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**: 1501–1512.
- THORNTON, B. R., and D. P. TOCZYSKI, 2003 Securin and B-cyclin/CDK are the only essential targets of the APC. *Nat. Cell Biol.* **5**: 1090–1094.
- TYERS, M., 1996 The cyclin-dependent kinase inhibitor p40SIC1 imposes the requirement for Cln G1 cyclin function at Start. *Proc. Natl. Acad. Sci. USA* **93**: 7772–7776.
- VERMA, R., R. S. ANNAN, M. J. HUDDLESTON, S. A. CARR, G. REYNARD *et al.*, 1997a Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science* **278**: 455–460.
- VERMA, R., R. M. FELDMAN and R. J. DESHAIES, 1997b SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. *Mol. Biol. Cell* **8**: 1427–1437.
- VISINTIN, R., S. PRINZ and A. AMON, 1997 CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science* **278**: 460–463.
- WITTENBERG, C., and S. I. REED, 2005 Cell cycle-dependent transcription in yeast: promoters, transcription factors, and transcriptomes. *Oncogene* **24**: 2746–2755.
- ZACHARIAE, W., and K. NASMYTH, 1999 Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev.* **13**: 2039–2058.
- ZACHARIAE, W., M. SCHWAB, K. NASMYTH and W. SEUFERT, 1998 Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. *Science* **282**: 1721–1724.

Communicating editor: P. RUSSELL